UNIVERSITI PUTRA MALAYSIA

STUDIES OF THE ANTI-CANCER EFFECTS OF FLAVOKAWIN B ONHUMAN BREAST CANCER CELL LINES, MCF7 AND MDA-MB-231

AJANTHA SINNIAH

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By

AJANTHA SINNIAH

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the requirements for the degree of Master's of Science

MARCH 2005

This thesis is especially dedicated to:

Amma & Appa, who are infinitely precious to me

&

Anu, Aravind and Abirami, who have filled my life with joy and happiness

&

My friends, who were there for me!

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master's of Science

STUDIES OF THE ANTI-TUMORIGENIC EFFECTS OF FLAVOKAWIN B ON HUMAN BREAST CANCER CELL LINES, MCF-7 AND MDA-MB-

By

AJANTHA SINNIAH

MARCH 2005

Chairman: Ahmad Bustamam Abdul, PhD

Faculty: Medicine and Health Sciences

A natural compound, Flavokawin B, isolated and purified from extract of *Alpinia zerumbet* was investigated for its anti-cancer properties on breast cancer cell lines, estrogen dependant MCF-7 and estrogen non-dependant MDA-MB-23. Tamoxifen, a non-steroidal anti-estrogen, primarily exploited as a drug against hormone-dependent breast cancer, acts as the positive control for this study. MCF-10A, mammary epithelial cells serve as the negative control. The cytotoxicities of Flavokawin B and Tamoxifen on human breast cells were investigated using the MTT assay. The results showed that the IC_{50} ($\pm$ S.E.M) value of Flavokawin B on MCF-7 cell line was determined to be 11.5 $\pm$ 0.015 μM/ml whilst the IC_{50} with Tamoxifen was at 10.2 $\pm$ 0.012 μM/ml. The IC_{50} value of Flavokawin B on MDA-MB-231 cell line was determined to be 17.5 $\pm$ 0.019 μM/ml whilst the IC_{50} value

of Tamoxifen was at 32.5 ± 4.2 μM/ml. The MTT assay results on normal epithelial cell line, MCF-10A treated with Flavokawin B demonstrated that the IC_{50} value was 38.0 ± 0.032 μM/ml whereas MCF-10A treated with Tamoxifen had an IC_{50} value of 28 ± 0.021 μM/ml. All values were statistically significant ($p<0.05$), as analysed using one sample T-test. The breast cancer cell lines treated at IC_{50} concentration of both compounds before proceeding using confocal microscopy. There were no significant changes observed in the untreated cells. However, apoptotic features were that include membrane blebbing and nucleus condensation were evident at 24 hours. At 48 and 72 hours post treatment, convolution of nuclear membrane, destruction of nuclear membrane and fragmentation of the nucleus were observed. The TUNEL assay is designed to specifically detect and quantify apoptotic cells within a cell population, which primarily consists of both apoptotic and non-apoptotic cells. The TUNEL assay conducted showed that Flavokawin B induces more apoptosis on MCF-7 and MDA-MB-231 compared to Tamoxifen. In contrast, Flavokawin B has lesser lethal effects on MCF-10A as compared to Tamoxifen. The levels of IL-6 secretion in MDA-MB-231 cell line decreased significantly after treatment with Flavokawin B. Immunofluorescence studies demonstrated that the levels of IL-6 secretion commensurate with the presence of membrane bound IL-6r when proliferation of the breast cells was inhibited during treatment with both the compounds. The MCF-7 and MDA-MB-231 cell lines were arrested at G1 phase when treated with both Flavokawin B

and Tamoxifen. This shows that both the treatment follows similar mechanism to induce cell phase arrest. In conclusion, it could be confirmed that the pure compound Flavokawin B induces apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines contributing to the discovery of new alternative treatment strategy for breast cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN ANTI-TUMORIGENIK FLAVOKAWIN B KE ATAS SEL-SEL SELANJAR PAYUDARA MCF-7 DAN MDA-MB-231

Oleh

AJANTHA SINNIAH

MARCH 2005

Pengerusi: Ahmad Bustamam Abdul, PhD

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Sebatian semulajadi Flavokawin B yang diasingkan dan ditulinkan daripada ekstrak Alpinia zerumbet telah dikaji bagi menentukan fungsi sebagai antikanser terhadap sel-sel selanjar payudara, samada bergantung kepada estrogen MCF-7 atau tidak bergantung kepada estrogen MDA-MB-231. Tamoxifen, anti-estrogen bukan steroid yang digunakan sebagai drug ke atas kanser payudara bergantung kepada estrogen digunakan sebagai kawalan positif bagi kajian ini. MCF-10A yang merupakan sel selanjar epithelial payudara digunakan sebagai kawalan negatif. Asai MTT digunakan untuk mengkaji kesan sitotoksik rawatan. Keputusan menunjukkan bahawa nilai IC_{50} (±S.E.M) untuk rawatan Flavokawin B ke atas sel selanjar MCF-7 ditentukan sebagai 11.5 ± 0.015 μM/ml sementara nilai IC_{50} bagi rawatan Tamoxifen ialah 10.2 ± 0.012 μM/ml. Nilai IC_{50} bagi Flavokawin B ke atas sel selanjar MDA-MB-231 ditentukan sebagai 17.5 ± 0.019 μM/ml

sementara nilai IC_{50} bagi rawatan Tamoxifen ialah 32.5 ± 4.2 μM/ml. Keputusan asai MTT ke atas sel selanjar MCF-10A menunjukkan nilai IC_{50} bagi Flavokawin B ialah 38.0 ± 0.032 μM/ml sementara nilai IC_{50} bagi rawatan Tamoxifen ialah 28 ± 0.021 μM/ml. Semua nilai IC_{50} adalah signifikan setelah dianalisis menggunakan satu sampel T-test (P <0.05). Kajian miksroskop konfokal diteruskan bagi semua sel-sel selanjar dirawat dengan Flavokawin B dan Tamoxifen pada kepekatan IC_{50} masing-masing. Tiada perubahan yang signifikan dilihat pada kumpulan kawalan. Walaubagaimanapun, ciri-ciri apoptosis telah dilihat seperti pengembungan membran dan kondensasi nukleus pada 24 jam. Pada 48 dan 72 jam selepas rawatan, konvolusi membran nucleus, pemusnahan dan fragmentasi membran nukleus telah dapat dilihat. Asai TUNEL telah direka untuk mengesan dan mengira sel-sel apoptotic dalam satu kumpulan sel yang terdiri daripada sel apoptotic dan bukan apoptotic. Berdasarkan keputusan, Flavokawin B merangsang lebih banyak apoptosis kepada MCF-7 dan MDA-MB-231 berbanding dengan rawatan Tamoxifen. Walaubagaimanapun, Flavokawin B kurang menghasilkan kesan kematian kepada sel selanjar MCF-10A berbanding dengan rawatan Tamoxifen. Tahap IL-6 bagi sel selanjar MDA-MB-231 berkurangan selepas dirawat dengan Flavokawin B. Kajian immunofluorescence menunjukkan bahawa tahap rembesan IL-6 bergantung kepada kewujudan IL-6r yang terdapat pada membran sel apabila pertumbuhan sel kanser terbantut ketika dirawat dengan kedua-dua rawatan tersebut. Sel selanjar MCF-7 dan MDA-MB-231

ditahan pada fasa G1 apabila dirawat dengan Flavokawin B dan Tamoxifen. Ini menunjukkan bahawa kedua-dua rawatan mempunyai mekanisma yang sama untuk menahan sel pada fasa tersebut. Kesimpulannya, sebatian semulajadi Flavokawin B merangsang apoptosis ke atas sel-sel selanjar MCF-7 dan MDA-MB-231 membawa kepada penemuan rawatan alternatif baru bagi rawatan kanser payudara.

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I certify that an Examination Committee met on 9th March 2005 to conduct the final examination of Ajantha Sinniah on her Master of Science thesis entitled "Anti-cancer Effects of Flavokawin B on Human Breast Cancer Cell Lines, MCF-7 and MDA-MB-231" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master's of Science. The members of the Supervisory Committee are as follows:

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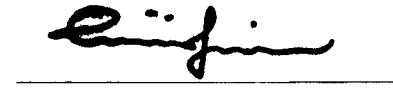

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Date: 15 JUL 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AJANTHA A/P SINNIAH

Date: 17 JUNE 2005

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

FCS	Fetal Calf Serum
IC	Inhibition Concentration
MTT	Microculture Tetrazolium Assay
IL-6	Interleukin 6
PBS	Phosphate Buffered Saline
rpm	rotation per minute
SPSS	Statistical Package for Social Sciences
ER	estrogen receptor
AO	Acridine orange
PI	Propidium iodide
ATCC	American Type Culture Collection
DMSO	Dimethyl sulphoxide
DNA	Deoksiribonucleic acid

CHAPTER 1

INTRODUCTION

Cancer is a genetic disease that undergoes clonal evolution of transformed cells that arise through the accumulation of mutations; either inherited (germline) or acquired (somatic), in critical proto-oncogenes and tumour suppressor genes. Carcinogens may be chemical, physical or biological in nature and interacts directly or indirectly with DNA and they are ubiquitous.

In countries such as Europe, USA, Canada, South America, breast cancer represents 25–30% of the total incidence of cancers in women and accounts for 15–18% mortality. The risk of a woman developing breast cancer during her lifetime is 1 in 8 in the United States, 1 in 12 in the European Community and 1 in 80 in Japan. Two-thirds of breast cancers are detected in postmenopausal women. Most breast cancers (about 95%), whether in pre- or postmenopausal women, are initially hormone-dependent, where the hormone estradiol plays a crucial role in their development and progression. The hormone and estrogen receptor (ER) complex can mediate the activation of proto-oncogenes and oncogenes (Pasqualini, 2004).

There are several new approaches towards cancer therapy. Breast cancer is estrogen responsive and is treated by hormonal therapy using Tamoxifen, an

anti estrogenic drug. Anti-cancer drugs used in chemotherapy, destroy cancer cells and these drugs work by interfering with the ability of cancer cells to divide and reproduce itself. The affected cells thus become damaged and eventually die. Unfortunately, most chemotherapeutic drugs also affect normal cells. The traditional approach in cancer therapy aimed at improving the overall survival of metastatic breast cancer include multiple lines of non-cross-resistant hormonal therapies, increasing the duration, the dose, and the dose intensity of chemotherapy, the use of non-cross-resistant polychemotherapy, and the addition of maintenance hormonal therapy. Thus far, the results have not been very rewarding despite prolongation of time to progression, improvements in overall survival were difficult to obtain, suggesting that these strategies do little to alter the natural history of breast cancer once it has metastasized (Awada, *et al.*, 2003).

The scientific evidence that plant based diets, in particular those rich in vegetables and fruits, protect against cancers of various sites has been found to be strong and consistent (Marchand, 2002). Flavonoids, which are structurally similar to estrogens, are able to bind to the estrogen receptor and possess either estrogenic or anti-estrogenic activities (Bail, *et al.*, 1998).

Elimination of tumour cells by the induction of apoptosis has become an important and new approach in cancer therapy. Apoptosis known as genetically programmed physiological form of cell death is not only involved in the

development of tumours but also plays an essential role in their treatment (Noteborn, *et al.*, 1998). Most of these bioactive substances exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumour cells has become an indicator of the tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Paschka,, *et al.*, 1998).

Recently natural plant researches have been contributing to drug innovation by providing plant derived anti-cancer agents. Since, nature has been provided with many effective anticancer agents, clinical plant based research has made progress in anticancer therapies (De Smet, 1997).